Short 5′-phosphorylated double-stranded RNAs induce RNA interference in *Drosophila*

Alexandra Boutla*†, Christos Delidakis*†, Ioannis Livadaras*, Mina Tsagris*† and Martin Tabler*

Double-stranded (ds) RNA causes the specific degradation of homologous RNAs in a process called "RNA interference (RNAi)" [1–4]; this process is called "posttranscriptional gene silencing (PTGS)" in plants [5–7]. Both classes of gene silencing have been reviewed extensively [8–13]. The duplex RNA becomes processed by Dicer [14] or another RNase III-like enzyme to short dsRNA fragments of about 21–23 nucleotides (nt) [15], which are incorporated in the RNA-induced silencing complex (RISC) [16] that directs target-specific RNA degradation [17, 18]. Here, we show that different synthetic dsRNA cassettes, consisting of two 5′-phosphorylated RNA strands of 22 nt each, can initiate RNAi in *Drosophila* embryos. The cassettes were active at similar quantities required to initiate RNAi by conventional dsRNA. Their sequence specificity was confirmed using synthetic dsRNA cassettes for two different genes, *Notch* and *hedgehog*; each time, only the relevant embryonic phenotype was observed. Introduction of point mutations had only a moderate effect on the silencing potential, indicating that the silencing machinery does not require perfect sequence identity. 5′-phosphorylated synthetic RNA was more active than its hydroxylated form. Substitution of either RNA strand by DNA strongly reduced activity. Synthetic cassettes of siRNA will provide a new tool to induce mutant phenotypes of genes with unknown function.

Addresses: *Institute of Molecular Biology and Biotechnology, Foundation for Research and Technology, P.O. Box 1527, GR-71110 Heraklion/Crete, Greece. †Department of Biology, University of Crete, GR-71110 Heraklion/Crete, Greece.

Correspondence: Martin Tabler
E-mail: tabler@imbb.forth.gr

Received: **6 August 2001**
Revised: **30 August 2001**
Accepted: **27 September 2001**

Published: **13 November 2001**

Current Biology 2001, 11:1776–1780

0960-9822/01/$ – see front matter

Results and discussion

Induction of RNA silencing by short synthetic dsRNAs

Recently, Elbashir et al. [19, 20] demonstrated that short synthetic RNAs can mediate RNA interference and sequence-specific RNA degradation in an in vitro system as well as in insect and mammalian tissue culture cells. For this class of short RNAs, they coined the term "short interfering RNAs (siRNAs)". We intended to test whether similar RNAs can induce RNAi in a whole organism and how their efficiency compares with conventional dsRNA. As the first target, we chose the *Notch* gene, which is ubiquitously expressed in the early embryo and whose loss of function produces a characteristic "neurogenic" phenotype [21]. The expressivity of the neurogenic phenotype can be used as a rough quantitative estimate of the severity of *Notch* function disruption.

An in vitro-synthesized 985-bp dsRNA fragment of the *Notch* mRNA was injected into *Drosophila* precellular embryos at 5 μM, which is a typical concentration for RNAi [4], of which about 100 pl, equivalent to about 0.5 fmole, were actually transferred. The dsRNA induced a strong *Notch* phenotype with high penetrance (Table 1a), indicative of an almost complete inactivation of the *Notch* mRNA, both zygotic and maternal [22] (Figure 1g,h).

Next, we synthesized two *Notch*-specific RNAs of 22 nucleotides, as shown in Table 1b. Selection of the sequence within the 985-bp cDNA fragment was based solely on structural considerations, to avoid self-dimerization or undesired intramolecular basepairing of each RNA molecule. The sequence was checked with BLAST [23] to ensure that it would interfere only with the *Notch* gene. In contrast with previous methods [19, 20], we used a simple all-RNA cassette, without deoxynucleotides in the 3′ protruding ends and without special considerations of which nucleotide would form the 3′ end. However, we additionally introduced a 5′ phosphate as an authentic RNase III product [24] and compared it with the nonphosphorylated RNA for its silencing potential. Both cassettes were adjusted to 100 μM and were used for injection. It should be noted that this is a 20-fold higher molar concentration than that of the dsRNA; however, in terms of absolute amount of RNA, it is less than half. The phosphorylated cassette was able to induce a strong *Notch* phenotype (Table 1b), exactly as observed after the injection of the long dsRNA. The nonphosphorylated cassette gave phenotypes with decreased penetrance, but the expressivity remained strong (Table 1c). At present, we

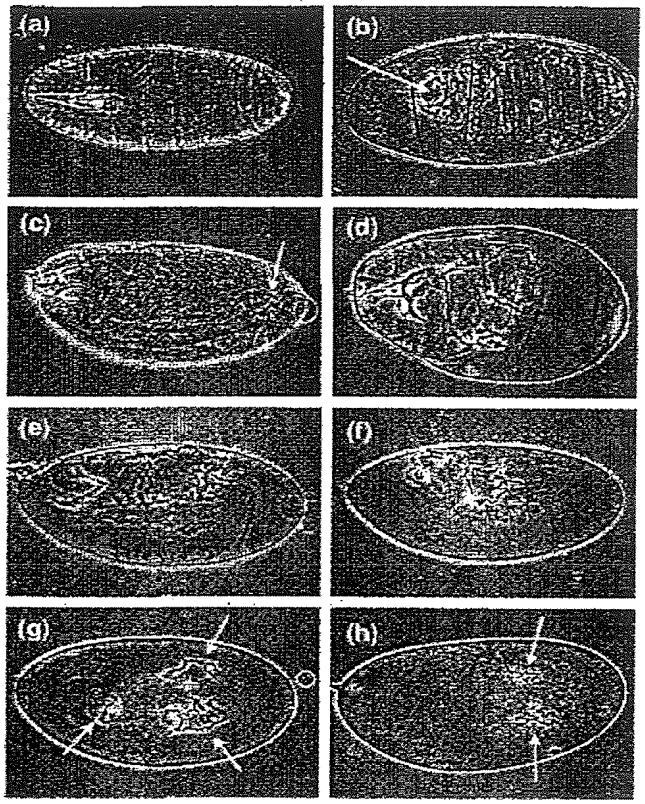
Table 1

Nucleic acids injected and their effect on induction of the *Notch* phenotype.

Nucleic acids injected[a]	Concentration[b]/ amount[c]	*Notch* phenotype		Other phenotype
		Percent penetrance (embryo #)[d]	Expressivity[e]	Viable/Unspecific[f]
(a) Notch dsRNA 985 bp	5/330	95 (384)	+++++	2/3
	1.65/110	55 (117)	++++	39/6
(b) R63: 5'-pACAAUGCUGCCUGCCACUACGA R61: GUUGUUACGACGGACGGUGAUGp-5'	100/140	92 (334)	+++++	3/5
	10/14	75 (278)	++++	16/9
(c) R63: 5'-HO-ACAAUGCUGCCUGCCACUACGA R61: GUUGUUACGACGGACGGUGAUG-OH-5'	100/140	65 (287)	+++++	33/2
(d) *D63: 5'-pACAATGCTGCCTGCCACTACGA* R61: GUUGUUACGACGGACGGUGAUGp-5'	100/140	15 (302)	++	66/17
(e) R63: 5'-pACAAUGCUGCCUGCCACUACGA *D61: GTTGTTACGACGGACGGTGATGp-5'*	100/140	23 (309)	++	48/29
(f) *D63: 5'-pACAATGCTGCCTGCCACTACGA* *D61: GTTGTTACGACGGACGGTGATGp-5'*	100/135	27 (301)	+	59/14
(g) R63: 5'-pACAAUGCUGCCUGCCACUACGA	100/70	1 (242)	+	70/29
(h) R61: 5'-pGUAGUGGCAGGCAGCAUUGUUG	100/70	12 (282)	+	70/18
(i) R63: 5'-pACAAUGCUGCCUGCCACUACGA R64: UGUUACGACGGACGGUGAUGCUp-5'	100/140	80 (322)	++++	19/1
	10/14	25 (213)	++	46/29
(j) R66: 5-'pACAAUGCUGUCUGCCACUACGA R65: GUUGUUACGACAGACGGUGAUGp-5'	100/140	81 (251)	+++++	19/0
	10/14	53 (261)	++	41/6
(k) R66: 5'-pACAAUGCUGUCUGCCACUACGA R61: GUUGUUACGACGGACGGUGAUGp-5'	100/140	88 (186)	+++++	12/0
(l) R63: 5'-pACAAUGCUGCCUGCCACUACGA R65: GUUGUUACGACAGACGGUGAUGp-5'	100/140	93 (245)	+++++	6/1
(m) R63: 5'-pACAAUGCUGCCUGCCACUACGA R67: GUUGUUACGACAAACGGUGAUGp-5'	100/140	21 (237)	++	49/30
	10/14	8 (297)	+	86/6
(n) Mock —	—/—	0 (216)	—	70/30
(o) Noninjected —	—/—	0 (123)	—	89/11

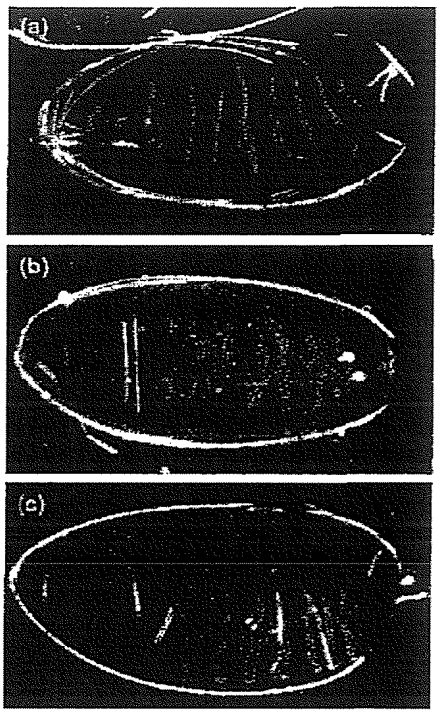
[a] The names and the sequences of the oligonucleotides are indicated; DNAs are given in italics; mutations are underlined; sense strond is on top.
[b] The concentration of the sample used for injection.
[c] This amount is calculated on the assumption that 100 pl is actually transferred.
[d] Numbers of embryos injected.
[e] This refers to *Notch* phenotypes (c)–(h) in Figure 1; +++++, almost exclusively strong phenotypes [(g),(h)]; ++++, strong phenotypes [(g), (h)] > intermediate phenotypes [(d)–(f)], ++, predominantly intermediate phenotypes [(d)–(f)]; +, exclusively weak phenotypes [(c)] or intermediate [(d)].
[f] These are embryos with various cuticle defects not attributable to loss of *Notch* function, as they are also seen in the control sample. Most frequent phenotypes are head holes and thin cuticle (see Figure 1b). It is noteworthy that the frequency of these presumably late defects (head involution and cuticle secretion) drops in the *Notch* RNAi samples. The likely reason is that the earlier *Notch* defect, which causes loss of cuticle, masks the subsequent manifestation of these phenotypes that are intrinsic to our fly population.

Figure 1



Examples of *Notch* phenotypes induced. Anterior is always to the left. **(a)** Almost wild-type embryo, ventral view. Seven ventral denticle belts are evident in the abdominal segments. **(b)** An example of an embryo with nonspecific effects. Note that although the segmented trunk is present, there is a hole in the head region (arrow). **(c–h)** Examples of *Notch* phenotypes: (c) weak phenotype with a small ventral hole (arrow). (d–f) Intermediate phenotypes; note that embryo (d) shows severe cuticle disruption in the posterior half (the site of injection), while the anterior half is almost wild-type. (g,h) Strong phenotypes with just small fragments of the dorsal cuticle remaining (arrows); such phenotypes were induced predominantly by long dsRNA and by phosphorylated siRNA cassettes. For a detailed view of which RNA induced which class of phenotype, see the explanation of footnote "e" in Table 1; for more information on *Notch*, see the Supplementary material available with this article online.

Figure 2



Examples of *hh* phenotypes induced by a synthetic dsRNA. The two oligonucleotides used were 5′-pUCCAUCUCCGCAAUCCCGC AAG-3′ and 5′-pUGCGGGAUUGCGGAGAUGGAGC-3′. The photograph was taken with dark field optics to make denticles prominent. Anterior is always to the left. **(a)** Ventral view of a wild-type embryo. Note the segmentally repeated denticle belts. **(b)** Ventral view, **(c)** lateral view of representative *hh* RNAi embryos. Note the absence of mouthpart structures and the coalescence of ventral denticles in an almost continuous lawn compared to the metameric pattern of the embryo in (a). The partial modulation in the width of denticle belts represents a vestige of segmentation, which is not due to incomplete inactivation of *hh*, as it is also observed in homozygous *hh* null embryos. For more information on *hh*, see the Supplementary material.

cannot distinguish whether the reduced efficiency is a general property or whether the phosphorylated 5′ terminus simply provided protection against exonucleases. It is noteworthy that the phosphorylated cassette had a higher penetrance at a 10-fold dilution compared to the undiluted nonphosphorylated dsRNA cassette (Table 1b,c), but under these conditions, its expressivity was slightly lower.

Induction of RNA silencing for the *hedgehog* gene

To test for the specificity and the general applicability of inducing RNAi, we used a second synthetic phosphorylated RNA cassette of the same general design, this time directed against the *hedgehog (hh)* gene (Figure 2). At a concentration of 100 μM, the *hh* dsRNA cassette induced a strong mutant phenotype in 88% of the 268 injected embryos. As for *Notch*, the strength of the observed phenotype suggested complete silencing of the *hh* gene.

RNA/DNA hybrids

According to current models, the antisense RNA confers sequence specificity upon the RNAi-mediated RNA degradation process [17, 25, 26]. In view of this, we next tested to what extent one of the RNA strands of the *Notch* siRNA cassette could be substituted by DNA. The combination DNA sense/RNA antisense was the most promising, since it left the antisense RNA intact. How-

ever, substitution of either sense or antisense strand by DNA resulted in a dramatic drop in both penetrance and expressivity of the *Notch* phenotypes (Table 1d,e) to levels comparable to those obtained with ordinary antisense RNA (Table 1h). A phosphorylated dsDNA cassette had an even weaker effect (Table 1f), inducing only a very mild *Notch* phenotype that had not been observed with any of the other samples tested (Figure 1c).

The influence of the termini of synthetic RNAs on RNA silencing

Subsequently, we tested whether the 3′ protruding ends, as generated by RNase III [24], are required. It was reported that blunt-ended RNAs were less active in insect tissue culture [20]. Further, Parrish et al. reported that a synthetic blunt-ended 26mer dsRNA was about 250-times less effective than an 81mer dsRNA [26], although a 26mer might be too large to act as a siRNA. In our case, we observed after injection with a blunt-ended RNA cassette an increased number of viable embryos (Table 1b,i) and, in accordance with this, a reduced expressivity. At 10-fold dilution, it became evident that this construct was less active than the proper siRNA. Thus, the protruding 3′ ends are not mandatory to elicit RNAi; although, in our case, the difference was not as pronounced as reported earlier. A potential difference to the blunt-ended cassette used previously [20, 26] is the presence of the 5′ phosphate in our construct.

The influence of mutations within synthetic RNAs on RNA silencing

Finally, we tested several RNA cassettes that carried mutations. In the first example, it was our intention to introduce a single nucleotide exchange that would interfere as much as possible with substrate binding. Therefore, the mutation was positioned centrally (Table 1j) and was simultaneously introduced into the sense and the antisense strand, so that the RNA cassette remained double stranded. In previous reports, nonmatching nucleotides greatly impaired the silencing potential when introduced to the antisense strand of longer dsRNAs [17, 26]. To our surprise, this synthetic cassette was also able to induce a strong *Notch* phenotype with high penetrance (Table 1j), indicating that a perfect match to the target RNA is not necessary to initiate the RNAi response. The 10-fold-diluted sample was still active, but penetrance and, in particular, expressivity were reduced. Next, we tested each of these mutated sense and antisense RNAs in combination with the wild-type sequence (Table 1k,l). Either of the combinations, characterized by a G:U pair or a mismatch, was highly active. As a third example, we tested an RNA cassette with a double mutation in the antisense strand paired with the nonmutated 22mer. Even this RNA construct, with its central bulge loop, had some silencing potential (Table 1m). However, both penetrance and expressivity dropped significantly compared with the single mutant. It will require a more detailed analysis to determine at what position and to what degree sequence deviations can be tolerated without loss of silencing function.

Conclusions

In this report, we have demonstrated that phosphorylated siRNAs can replace longer dsRNA to induce RNAi in an insect with comparable efficiency. During the review of this manuscript, Caplen et al. [27] reported that synthetic RNAs inhibit gene expression in *Caenorhabditis elegans*. However, the organisms differ in their response to synthetic RNA. While 89% of the progeny broods of dsRNA-treated worms showed a decrease in gene function, only 16.3% showed a decrease when a 25mer siRNA was used. Reduction of the nucleotide number to 24 and 23 reduced that portion to 3.6% and 1.4% (2 out of 145 animals), respectively. That is at variance with the situation that we observe for *Drosophila*, in which siRNAs have activity comparable to that of dsRNAs. A potential difference is the fact that we directly score the injected embryos. Further, we found that not all aspects of the RNase III product mimic are absolutely necessary. Neither the 3′ protruding ends nor the 5′ phosphate are mandatory, but both modifications are advantageous, since they strengthen the silencing potential of the synthetic RNA.

The observation that the inducing mechanism does not require perfect homology has consequences not only for the practical usage of synthetic cassettes in gene silencing, but it also allows us to envisage that there is crosstalk between genes. It is conceivable that a particular siRNA originating from a silenced gene may silence a second gene. An extensive overall sequence similarity of the two genes would not be necessary; short similar sequence domains of either polarity would be sufficient. This might turn out to be a naturally occurring way of gene regulation, especially during development. Further, in plants, an impact of short RNAs on transcriptional gene silencing (TGS) has been demonstrated. It was shown that the expression of dsRNA may result in promoter methylation [28, 29], and recent data suggest that short RNAs direct de novo methylation of homologous DNA [30]. In view of leaky sequence requirements for the induction of RNAi by short RNAs, it is possible that neither RNA-directed methylation requires strict sequence identity.

The use of synthetic RNAs to induce RNA silencing will drastically simplify approaches for the systematic generation of mutant phenotypes in order to assign gene function in *Drosophila*, such as that already carried out for *C. elegans* [31, 32]. It needs to be tested whether and to what extent synthetic RNA cassettes are capable of inducing gene silencing in other organisms, including mammals; however, our initial attempts to induce RNAi in mouse were not successful (unpublished data).

Supplementary material

Supplementary material describing Materials and methods and mutant phenotypes is available at http://images.cellpress.com/supmat/supmatin.htm.

Acknowledgements

We thank Mr. Dimitrios Alexandrou for subcloning a *Notch* cDNA fragment and Drs. Michela Denti, Kriton Kalantidis, and Kyriacos Petratos for critical reading of the text. This work was supported by the Greek Ministry of Development (Grants TDE97 EKBAN2#94 and PENED 99 ED 404).

References

1. Cogoni C, Macino G: **Isolation of quelling-defective (qde) mutants impaired in posttranscriptional transgene-induced gene silencing in *Neurospora crassa*.** *Proc Natl Acad Sci USA* 1997, **94:**10233-10238.
2. Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC: **Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*.** *Nature* 1998, **391:**806-811.
3. Montgomery MK, Xu S, Fire A: **RNA as a target of double-stranded RNA-mediated genetic interference in *Caenorhabditis elegans*.** *Proc Natl Acad Sci USA* 1998, **95:**15502-15507.
4. Kennerdell JR, Carthew RW: **Use of dsRNA-mediated genetic interference to demonstrate that frizzled and frizzled 2 act in the wingless pathway.** *Cell* 1998, **95:**1017-1026.
5. Waterhouse PM, Graham MW, Wang MB: **Virus resistance and gene silencing in plants can be induced by simultaneous expression of sense and antisense RNA.** *Proc Natl Acad Sci USA* 1998, **95:**13959-13964.
6. Chuang CF, Meyerowitz EM: **Specific and heritable genetic interference by double-stranded RNA in *Arabidopsis thaliana*.** *Proc Natl Acad Sci USA* 2000, **97:**4985-4990.
7. Papaefthimiou I, Hamilton A, Denti M, Baulcombe D, Tsagris M, Tabler M: **Replicating potato spindle tuber viroid RNA is accompanied by short RNA fragments that are characteristic of post-transcriptional gene silencing.** *Nucleic Acids Res* 2001, **29:**2395-2400.
8. Hammond SM, Caudy AA, Hannon GJ: **Post-transcriptional gene silencing by double-stranded RNA.** *Nat Rev Genet* 2001, **2:**110-119.
9. Sharp PA: **RNA interference–2001.** *Genes Dev* 2001, **15:**485-490.
10. Vance V, Vaucheret H: **RNA silencing in plants–defense and counterdefense.** *Science* 2001, **292:**2277-2280.
11. Voinnet O: **RNA silencing as a plant immune system against viruses.** *Trends Genet* 2001, **17:**449-459.
12. Waterhouse PM, Wang M, Lough T: **Gene silencing as an adaptive defence against viruses.** *Nature* 2001, **411:**834-842.
13. Matzke M, Matzke AJ, Kooter JM: **RNA: guiding gene silencing.** *Science* 2001, **293:**1080-1083.
14. Bernstein E, Caudy AA, Hammond SM, Hannon GJ: **Role for a bidentate ribonuclease in the initiation step of RNA interference.** *Nature* 2001, **409:**363-366.
15. Hamilton AJ, Baulcombe DC: **A species of small antisense RNA in posttranscriptional gene silencing in plants.** *Science* 1999, **286:**950-952.
16. Hammond SM, Bernstein E, Beach D, Hannon GJ: **An RNA-directed nuclease mediates post-transcriptional gene silencing in *Drosophila* cells.** *Nature* 2000, **404:**293-296.
17. Yang D, Lu H, Erickson JW: **Evidence that processed small dsRNAs may mediate sequence-specific mRNA degradation during RNAi in *Drosophila* embryos.** *Curr Biol* 2000, **10:**1191-1200.
18. Zamore PD, Tuschl T, Sharp PA, Bartel DP: **RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals.** *Cell* 2000, **101:**25-33.
19. Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K, Tuschl T: **Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells.** *Nature* 2001, **411:**494-498.
20. Elbashir SM, Lendeckel W, Tuschl T: **RNA interference is mediated by 21- and 22-nucleotide RNAs.** *Genes Dev* 2001, **15:**188-200.
21. Lehman R, Jimenez F, Dietrich U, Campos-Ortega JA: **On the phenotype and development of mutants of early neurogenesis in *Drosophila melanogaster*.** *Roux Arch Dev Biol* 1983, **192:**62-74.
22. Jimenez F, Campos-Ortega JA: **Maternal effects of zygotic mutants affecting early neurogenesis in *Drosophila*.** *Roux Arch Dev Biol* 1982, **191:**191-201.
23. Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, *et al.*: **Gapped BLAST and PSI-BLAST: a new generation of protein database search programs.** *Nucleic Acids Res* 1997, **25:**3389-3402.
24. Nicholson AW: **Function, mechanism and regulation of bacterial ribonucleases.** *FEMS Microbiol Rev* 1999, **23:**371-390.
25. Bass BL: **Double-stranded RNA as a template for gene silencing.** *Cell* 2000, **101:**235-238.
26. Parrish S, Fleenor J, Xu S, Mello C, Fire A: **Functional anatomy of a dsRNA trigger. Differential requirement for the two trigger strands in RNA interference.** *Mol Cell* 2000, **6:**1077-1087.
27. Caplen NJ, Parrish S, Imani F, Fire A, Morgan RA: **Specific inhibition of gene expression by small double-stranded RNAs in invertebrate and vertebrate systems.** *Proc Natl Acad Sci USA* 2001, **98:**9742-9747.
28. Mette MF, Aufsatz W, van der Winden J, Matzke MA, Matzke AJ: **Transcriptional silencing and promoter methylation triggered by double-stranded RNA.** *EMBO J* 2000, **19:**5194-5201.
29. Sijen T, Vijn I, Rebocho A, van Blokland R, Roelofs D, Mol JN, *et al.*: **Transcriptional and posttranscriptional gene silencing are mechanistically related.** *Curr Biol* 2001, **11:**436-440.
30. Mette MF, Matzke AJM, Matzke MA: **Resistance of RNA-mediated TGS to HC-Pro, a viral suppressor of PTGS, suggests alternative pathways for dsRNA processing.** *Curr Biol* 2001, **11:**1119-1123.
31. Fraser AG, Kamath RS, Zipperlen P, Martinez-Campos M, Sohrmann M, Ahringer J: **Functional genomic analysis of *C. elegans* chromosome I by systematic RNA interference.** *Nature* 2000, **408:**325-330.
32. Gonczy P, Echeverri G, Oegema K, Coulson A, Jones SJ, Copley RR, *et al.*: **Functional genomic analysis of cell division in *C. elegans* using RNAi of genes on chromosome III.** *Nature* 2000, **408:**331-336.